To Dr. Susan ...

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Court et al.
Application No. 10/692,553
Filed: October 23, 2003
Confirmation No. 1179
For: ENHANCED HOMOLOGOUS RECOMBINATION MEDIATED BY LAMBDA RECOMBINATION PROTEINS
Examiner: Jennifer Ann Dunston
Art Unit: 1636
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DECLARATION OF PENTAO LIU AND E-CHIANG LEE UNDER 37 C.F.R.§ 1.132

1. We, E-Chiang Lee and Pentao Liu are inventors of the above-identified patent application. It is our understanding that claims 1, 3-10, 12-13 and 22-23 have been rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Rajewsky et al. (J. Clin. Invest. 98(3) 600-303, 1996) in view of Lee et al. (Genomics 73: 56-65, 2001), which is the publication of the work of E-Chiang Lee and several of the other inventors.

Rajewsky et al. discloses that the genome in ES cells can be modified by conditional gene targeting using the Cre-loxP system. Rajewsky et al. describe a gene targeting vector that includes three loxP sites, two of which flank a nucleic acid encoding a selectable marker. The targeting vector is introduced into embryonic stem cells, and endogenous homologous recombination is used so that the genome of the ES cells includes the selectable marker flanked by two loxP sites and the additional loxP site in the genome of the host ES cell. The expression of Cre in the ES cells results in recombination at the loxP sites. In some of the ES cells, the selectable marker is deleted, and two loxP sites remain. These ES cells are then used to generate mice. The mice including the two loxP sites are mated with another mouse that expresses Cre in

specific cells of interest. Rajewsky et al. only teach (1) the production of vectors including two selectable markers and three loxP sites, wherein only two of the loxP sites flank a selectable marker; (2) the use of homologous recombination *not to produce vectors* but to introduce the vectors into ES cells, and (3) the production of mice from these ES cells. Rajewsky et al. teach that mice that include two loxP sites flanking a selectable maker can be mated to mice expressing Cre to produce gene knock-outs in animals. *There is no information in Rajewsky et al. with regard to methods for producing the vectors. Indeed, as the homologous recombination system described by Rajewsky et al. is only functional in eukaryotic cells, such as ES cells, Rajewsky et al. cannot even be construed to suggest using homologous recombination for the production of vectors in bacterial cells.*

Submitted herewith is a copy of Seibler et al., which describes the methods used to produce the constructs used by Rajewsky et al. It is clear that classical methods, such as PCR amplification, restriction digestion, ligation, and site directed mutageneiss were used to produce the vectors described by Rajewsky et al. In addition, these methods result in vectors that include one pair of loxP sites, flanking a single selectable marker.

Lee et al. describe the use of recombineering (the use of a de-repressible promoter operably linked to Beta Exo and Gam) to engineer bacterial artificial chromosomes. Recombineering is used to target a Cre gene to exon 2 of the Eno gene. The method for producing the targeting vector is described on page 61. Specifically, a IRES-eGFP-FRT-kan-FRT cassette was amplified using PCR. The 3' twenty-one nucleotides of each primer were homologous to the targeting cassette, and the 5' forty-two nucleotides were homologous to the last exon of Eno2 where the cassette was to be targeted. The primers were designed to target the cassette downstream of the Eno2 stop codon and upstream of the poly A site (thus to be in the 3' untranslated region). Homologous recombination was allowed to occur, and kanamycin resistant colonies were selected. The kan selectable marker was removed by expressing flpe in cells, thereby inducing recombination at the FRT sites. The construct results in the production of a construct that produces (delete) a FUNCTIONAL Cre protein. These vectors are not designed to produce knockouts.

The BAC vector used in these experiments included a LoxP site (see page 61). However, homologous recombination (recombineering) was used to remove this LoxP site in order to avoid un-desired recombination. Thus, targeting methods used in Lee et al. do use homologous recombination to insert a nucleic acid encoding a first selectable marker flanked by a pair of recombining sites (FRT) in to first site in a gene in a BAC, and to excise the nucleic acid encoding the selectable maker.

Indeed, the work described in Lee et al. is completely different from the presently claimed methods of producing vectors. The methods described in Lee et al. do not use a second set of recombining sites flanking a second selectable marker, and they do not result in a nucleic acid sequence that CANNOT be transcribed into a functional protein. Indeed, the intent of Lee et al. is to induce a functional nucleic acid encoding Cre into an untranslated region of a gene (Eno2), such that Cre is expressed in a tissue specific manner along with the Eno2 protein.

It is difficult to understand how Rajewsky et al. could be combined with Lee et al. to derive the presently claimed methods. Neither reference teaches or suggests: (1) the use of two selectable markers, each flanked by a pair of recombining sites; (2) the use of homologous recombination to make vectors for conditional knock-outs; or (3) specific steps in any method that would lead to a vector for a conditional knockout. Indeed, a large number of experiments were required to produce the claimed methods for producing vectors for conditional knockouts. Some of our data documenting the conception of the claimed methods has been presented in the Declaration of Dr. Liu under 37 C.F.R. § 1.132. As can be seen from this data, a large number of experiments were required to devise the presently claimed methods.

Stewart et al. and Muyers et al. teach alternative methods for homologous recombination in bacterial cells, describing cloning and subcloning and documenting that homologous recombination can be used to introduce a single selectable marker. There is no description in either Stewart et al. or Muyers et al. of specific steps in a method for generating conditional targeting vectors. However, these publications are silent with regard to any methods for producing vectors for conditional knock-outs, let alone (1) the use of two selectable markers, each flanked by a pair of recombining sites; (2) the use of homologous recombination to make

vectors for conditional knock-outs; or (3) specific steps in any method that would lead to a vector for a conditional knockout.

Thus, none of Rajewsky et al., Lee et al., Stewart et al. or Muyers et al. provide any means or suggestions of steps in a method that utilizes homologous recombination to generate vectors for conditional knock-out of a gene.

3. The present claimed methods provide a rapid and efficient method for generating cko-targeting vectors. This method uses recombineering rather than restriction enzymes and DNA ligases for vector construction (see the specification, Fig. 18). By using high copy plasmid DNA for vector construction, the problem caused by Lox sites present in the BAC vector backbone is eliminated (discussed in Rajewsky et al.). As many as 10,000 colonies can be obtained from a single subcloning experiment with only 50-100 ng of retrieving plasmid DNA. In addition, more than 95% of the colonies are correctly constructed. This could not be predicted, and is in contrast to previous subcloning methods, such as those methods likely used to produce the vectors disclosed in Rajewsky et al, see above. Moreover, using long homology arms, targeting frequencies as high as $1X10^{-2}$ can be obtained with as little as 100 ng of targeting DNA (such as when inserting a floxed *Neo* cassette to a BAC).

It is impossible to directly provide a side-by side comparison of the methods disclosed by Rajewsky et al., as the simply are no methods for the production of vectors for conditional knock-out of a gene in a cell disclosed in this reference. Moreover, addition the disclosure of Lee et al., Stewart et al. and/or Muyers et al. does not provide defined steps in a method, such that a side-by-side comparative analysis can be performed in the laboratory.

However, general conclusions can be drawn. As disclosed in the specification, using the presently claimed methods, all of the reactions needed to construct a conditional knock-out vector, including digestion of the PCR products and ligation and transformation, can be done in one day. Typically, it takes less than two weeks to construct a vector using the claimed methods and multiple vectors can be generated simultaneously. This is in stark contrast to classical methods for producing a vector for conditional knock-out of a gene, such as described in Seibel

et al. Generally, using classical molecular techniques, it takes several months to prepare just a single vector. The recombineering method allows one person to make a conditional knockout vector and several vectors simultaneously in 2-3 weeks. The preparation of multiple vectors is not an option using classical methods.

4. We hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of the Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Pentao Liu, Ph.D. — Date: Nov. 9, 2008

E-Chiang Lee, Ph.D. — Date: Nov. 11, 2008